The effects of feeding and overwintering conditions on emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) energy reserves and flight performance

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Abstract

The emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) is an invasive beetle from Asia that has been confirmed in 30 states as of July 2017. The spread of *A. planipennis* has been markedly slower in Minnesota than in other states. Cold winter temperatures are thought to be the primary factor for the slower spread. The goals of this study were to determine the role of adult feeding on *A. planipennis* energy reserves and flight capacity, as well as to elucidate any sub-lethal effects of winter conditions on *A. planipennis* flight capacity. In 2015 and 2016, adult *A. planipennis* were reared from infested green ash logs collected in Hennepin and Ramsey Counties, MN. A separate experiment was conducted by collecting infested logs from St Paul, MN and stored in two locations during the winter of 2015-2016. Grand Rapids, MN, and St Paul, MN, to determine how winter conditions affect *A. planipennis* energy reserves and flight capacity. Adults were individually placed in cages and provided with fresh, lab-grown shamel ash (*Fraxinus uhdei* Wenzig) leaves on which to feed for 0-20 days before being flash frozen or flown on a custom flight mill for 24 hours under constant light. Beetles were subjected to nutrient analysis using either petroleum ether or colorimetric assays. Feeding treatments were compared for weight and lipid gain, flight velocity and total distance flown.

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Introduction

The emerald ash borer (*Agrilus planipennis* Fairmaire) is estimated to have killed tens of millions of North American ash (*Fraxinus* spp.) trees since its initial detection in the United States and Canada nearly 15 years ago (McCullough et al. 2009). As *A. planipennis* has spread into regions with sub-optimal climates, there will likely be areas where *A. planipennis* populations experience physiological stress, which may alter the spread of the beetle. This review examines the introduction of *A. planipennis* into North America, the beetle's life history, dispersal behavior, and energetic costs associated with overwintering and flight.

Introduction of Emerald Ash Borer

Agrilus planipennis is a Buprestid beetle native to eastern Asia, ranging from northeastern China and far eastern Russia, south to Taiwan, and east to Japan (Haack et al. 2002, Liu et al. 2003, Poland and McCullough 2006). *Agrilus planipennis* was discovered outside of Detroit, Michigan in June 2002; however, dendrological evidence suggests that the insect likely established in the United States as early as the mid-1990s (Cappaert et al., 2005; Poland & McCullough, 2006; Siegert et al., 2014). The insect was likely introduced through shipping of untreated wooden pallets and crates (Haack et al. 2002, Herms et al. 2004).

The host range of *A. planipennis* includes many species in the family Oleaceae, with a noted preference for ash trees (*Fraxinus* spp.) (Liu et al. 2003, Zhao, T.H., Gao, R.T., Liu, H.P., Bauer, L.S. and Sun 2005, Anulewicz et al. 2008). *Agrilus planipennis* is generally only a minor pest in its native range and only kills stressed or weakened trees

(Zhao et al., 2005). However, most North American ash species have no effective resistance to *A. planipennis*, allowing any tree to be infested (Eyles et al. 2007, Chen et al. 2011, Koch et al. 2015, Rigsby et al. 2015). Healthy trees can be killed by *A. planipennis* in as little as three years (Herms et al. 2004). From its initial establishment in the United States and Canada, *A. planipennis* has spread more rapidly to the east and south than to the north and west. Currently, *A. planipennis* has been confirmed in 30 US states and southern portions of Ontario and Quebec (USDA, 2017; NRC, 2017).

Although adult *A. planipennis* are strong flyers, there is evidence that the spread of *A. planipennis* has been aided through human-mediated dispersal (Cappaert et al. 2005, Mercader et al. 2009, Taylor et al. 2010). Ash is frequently cut and used as firewood. In instances where firewood is not properly sanitized, *A. planipennis* larvae can continue development and emerge as adults from the cut wood after transport (Sobek et al. 2011).

Ash species were commonly planted in urban areas in the 1970s as replacements for dying elm trees as Dutch elm disease spread throughout the United States (Poland and McCullough 2006). Ash was a preferred urban tree due to its fast growth, high shade production, and relatively low debris. The arrival and spread of *A. planipennis* has incurred significant costs to urban areas (Sydnor et al. 2007, Kovacs et al. 2010). Unlike elm species, ash trees become very brittle after death and are prone to dropping large limbs and falling into roads or on property (Ball et al. 2007). Management efforts must be implemented on boulevard ash trees once *A. planipennis* is established in an area. The two primary options for management involve either removal (and potentially

replacement) of the tree or the injection of systemic insecticides (Sydnor et al. 2007, Kovacs et al. 2010, Herms et al. 2014). These programs can be costly, with estimated costs for removal and replacement of urban trees alone ranging $10- 13 billion, over 10 years (Kovacs et al. 2010, Sydnor et al. 2011). Biological control programs, typically involving the release of parasitoid wasps, are also being implemented in certain areas (Duan et al. 2012).

Agrilus planipennis presents further ecological risks. Over 200 arthropod species are highly associated with *Fraxinus spp*. These species may risk extirpation if host trees are lost due to *A. planipennis* attack (Gandhi and Herms 2010, Wagner and Todd 2016). There is also concern for the environmental impact in the north-central portion of Minnesota, where black ash (*F. nigra* Marshall) is the predominant tree species in poorly drained soils (Telander 2013). The loss of black ash in this region could potentially alter the hydrology of these wetlands, and have a cascading effect on the ecosystem (Youngquist et al. 2017).

Life History of Emerald Ash Borer

Agrilus planipennis is typically univoltine, although the insect does have a semivoltine lifecycle in colder climates within its native range (Wei et al. 2007). After hatching in the summer, *A. planipennis* larvae bore through the bark of the host tree and begin feeding on phloem and cambium (Wei et al., 2007; Wang et al., 2010). Larval feeding is the causal agent of damage to trees, as serpentine galleries effectively girdle the stem of the trees (Cappaert et al. 2005, Poland and McCullough 2006, Wei et al. 2007). Larvae undergo four feeding instars during the summer (Wei et al. 2007, Wang et

al. 2010, Poland et al. 2015). In the fall, mature larvae excavate pupal chambers in the outer sapwood in which to overwinter. Larvae in semivoltine populations will overwinter as second or third instars in feeding galleries. Larvae pupate the following spring, once temperatures rise. Pupation typically lasts 4-8 weeks, or 250 degree days above a base of 10° C (Brown-Rytlewski and Wilson 2004, Wei et al. 2007, Wang et al. 2010, Poland et al. 2015). Adults emerge and climb into the canopy of their host tree and feed on foliage for a few days before dispersal (Rodriguez-Saona et al. 2007, Wei et al. 2007).

Flight is important to the mating behavior of *A. planipennis*. Males typically fly around ash trees, either circling above the canopy or hovering around the main stem, to find mates (Lelito et al. 2007, Rodriguez-Saona et al. 2007). Females that are ready to mate will make themselves apparent, often resting on leaflets in the upper canopy (Lelito et al. 2007, Wei et al. 2007). There are no courtship behaviors before copulation. A contact sex pheromone produced by females signals males to initiate mating (Lelito et al., 2009; Rodriguez-Saona et al., 2007; Silk et al., 2009).

The observed mating behaviors of *A. planipennis* may help explain flight behavior during tethered flight studies, where males fly significantly longer durations of time than virgin females and mated females fly nearly 2.5 times farther than virgin females (Taylor et al., 2010). Early flight mill studies found that *A. planipennis* are fairly strong flyers, capable of reaching speeds over 1.5 m/sec while on tethers (Taylor et al., 2005). *Agrilus planipennis* typically exhibit short bursts of flight, lasting about 1 min, followed by periods of inactivity (Taylor et al., 2005; Taylor et al., 2010). Unmated *A. planipennis* are

capable of flying just over 1 km, on average, while some individuals are capable of flying over 6 km (Fahrner et al. 2015).

Reconstructions of spread of *A. planipennis* show that the beetles likely fly less than their ultimate capacity, as spread rates average 3.4 km/yr (Siegert et al. 2014). Extra-range dispersal is likely caused by human-mediated movement of infested materials, such as firewood (Cappaert et al. 2005, Poland and McCullough 2006). The localized spread of *A. planipennis* is largely determined by female foraging behavior and the surrounding environment (Siegert et al., 2010; Mercader et al., 2011). Female *A. planipennis* selectively oviposit on stressed trees, but avoid areas where phloem resources are depleted (McCullough et al., 2009; Siegert et al., 2010). While resources are readily available, females often oviposit within 100 m of their host tree, but females are capable of dispersing much greater distances if necessary (Mercader et al., 2009; Taylor et al., 2010; Mercader et al., 2011; Fahrner et al., 2015).

Cold Tolerance

Insects that live in temperate climates must find ways to confront the cold temperatures of winter months (Lee 1989, Sinclair et al. 2003). *Agrilus planipennis* has been found to exhibit freeze intolerance as a strategy to survive cold temperatures. *Agrilus planipennis* larvae rapidly increase glycerol concentration in hemolymph beginning in November, with highest concentrations being achieved in December, and remaining high until the following spring (Crosthwaite et al. 2011). If overwintering *A. planipennis* prepupae are exposed to temperatures above freezing for extended periods, glycerol concentrations in hemolymph decrease, resulting in an irreversible loss of cold

tolerance (Sobek-Swant, Crosthwaite, et al. 2012). Levels of other polyols, such as sorbitol, have been found to rapidly decline during exposure to temperatures above 0° C, being restored to glycogen reserves, while glycerol persists for a number of days before being metabolized (Storey and Storey 1983). *Agrilus planipennis* has been found to have a mean super cooling point (i.e., SCP, defined as the temperature at which ice forms inside the insect body) of -25° C, with the lowest SCP recorded being -35.3° C (Venette and Abrahamson 2010, Crosthwaite et al. 2011). There is also a thermal buffering effect of tree bark, which typically keeps overwintering larvae 4° C warmer than ambient air temperatures (Vermunt et al. 2012). Using climatological data, DeSantis et al. (2013) estimated that central Minnesota is the northern limit that can support populations of *A. planipennis* sufficient to cause widespread tree mortality.

Freeze intolerant insects can survive temperatures below freezing by reducing their SCP. Insects that are freeze intolerant must undergo biological and physiological shifts to prevent ice crystallization, such as increasing lipid content, reducing water content, and/or evacuating gut contents after feeding to reduce ice-nucleating agents (Neven et al. 1986; Pullin et al. 1987; Bale 2002). Many insects also produce and release numerous molecules that act as antifreeze compounds (Storey and Storey 1983, Storey and Storey 2012, Arrese and Soulages 2010). There are numerous proteins and polyols that can be released into hemolymph that can significantly lower the freezing point of the insect's body fluids. The most common polyols are glycerol, sorbitol and to a lesser degree, xylitol (Storey and Storey 1983, Storey 1997, Crosthwaite et al. 2011). Glycogen is commonly broken down into glycerol, as well as the disaccharide trehalose, which, in

addition to serving as an energy source, also functions as an antifreeze compound in insect hemolymph (Steele 1982, Becker et al. 1996, Storey 1997).

Although glycerol can be sourced from lipids, it is most commonly derived from glycogen (Storey 1997, Lorenz and Anand 2004, Arrese and Soulages 2010). Lipid is anhydrous while glycogen has significant water content. Thus, metabolizing glycogen aids in water reduction during overwintering (Arrese and Soulages 2010). The metabolization of lipid also releases water, which, while harmful during winter, aids in rehydration the following spring (Downer and Matthews 1976, Arrese and Soulages 2010).

Many insects rapidly increase lipid content during their last larval instar, especially insects that overwinter in this stage (Timmermann and Briegel 1999, Lorenz and Anand 2004, Anand and Lorenz 2008). Lipids are heavily utilized during periods of starvation, and during periods of high energy demand, such as sustained flight (Beenakkers et al. 1984, 1985, Ziegler 1991, Athenstaedt and Daum 2006). Many insects rely on lipid reserves for long distance dispersal, as lipid metabolism produces more energy per unit weight (Downer and Matthews 1976).

With energy reserves being essential to a number of life processes, tradeoffs have been documented when energy reserves are partitioned amongst life events, especially dispersal and reproduction (Chaplin and Wells 1982, Zera and Larsen 2001). Monarch butterflies, for example, lose substantial amounts of lipid while overwintering, and must replenish energy reserves before migrating north (Chaplin and Wells 1982). Since many insects are unable to synthesize lipids as adults, lipid is a more valuable resource, while

glycogen can be obtained through adult feeding (Chaplin and Wells 1982, Lorenz and Anand 2004, Anand and Lorenz 2008).

Conclusion

Minnesota may be at the northern edge of the potential range for *A. planipennis* (Poland and McCullough 2006, Sobek-Swant et al. 2012, USDA Forest Service Forest Health Technology Enterprise Team 2012, DeSantis et al. 2013). After initial detection in the state of Minnesota in 2009, the spread of *A. planipennis* has been slower compared to other states. Nevertheless, populations continue to build, and the range of *A. planipennis* is expanding, primarily around the Twin Cities region and the southeastern portion of the state. Winter temperatures have been highly variable in recent years, with generally warmer-than-average temperatures, followed by cold events where temperatures reach below-30° C. This stochastic winter climate may have lasting detrimental effects for *A. planipennis* that manage to survive to adulthood.

My thesis explores the effects of different winter conditions on *A. planipennis* energy reserves (defined as lipid, glycogen, and sugars), as well as how adult maturation feeding affects these energy reserves. Flight mill studies are also performed to determine if winter conditions have any impact on the flight potential of *A. planipennis*. This information may prove useful in determining the rate of spread in areas that are less environmentally favorable for *A. planipennis*, which may also aid in management options. My data chapter is written as a stand-alone publication suitable for a peer-reviewed journal. As such, a small degree of redundancy with this introduction may be present to ensure the integrity of the paper as a stand-alone unit. Although this work was

performed by myself in pursuit of a graduate degree, I used plural voice in places to acknowledge the contributions of my coauthors consistent with forthcoming submission to a peer-reviewed journal.

CHAPTER 1

Effects of feeding and overwintering location on *Agrilus planipennis* energy reserves and flight performance

I. Introduction

Emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is a phloem feeding beetle that was first detected in North America in 2002, near Detroit, Michigan and Windsor, Ontario (Poland and McCullough 2006). *Agrilus planipennis* is a severe pest of native North American ash species (*Fraxinus spp.*) and often attacks healthy trees (Haack et al. 2002). North American ash species have little to no resistance to *A. planipennis* attack (Eyles et al. 2007, Rigsby et al. 2015). Black ash (*F. nigra* Marshall) and green ash (*F. pennsylvanica* Marshall) are the two most commonly occurring species in Minnesota. Black ash is common in northern Minnesota, often occurring in pure stands (Iverson et al. 2016). Ash species, particularly green ash, are commonly planted in urban areas, and break-up can pose significant public safety risks after *A. planipennis* kills trees. Estimated costs of removal and replacement of urban ash trees in North America range from $5.6- $11 B (Kovacs et al. 2010, Sydnor et al. 2011, McKenney et al. 2012).

Tree damage occurs as *A. planipennis* larvae feed on phloem, with numerous larval galleries eventually girdling the host tree (Cappaert et al., 2005). In the fall, late instar larvae stop feeding and excavate pupal chambers in the outer sapwood in which to overwinter. Once temperatures rise in the spring, larvae remain in place to pupate (Cappaert et al. 2005). Pupation lasts approximately 30 days before adults emerge and climb into the canopy for maturation feeding on foliage for a few days before mating and

dispersal (Rodriguez-Saona et al. 2007; Wei et al. 2007; Wang et al. 2010). Since no feeding occurs from October-November until adult emergence the following year when temperatures allow for development, *A. planipennis* larvae have to rely on energy reserves (e.g., glycogen and lipids) obtained over the previous summer to successfully overwinter and pupate.

Both overwintering and pupation have significant energetic costs. *Agrilus planipennis* is a freeze-intolerant insect; in order to prevent freezing it releases glycerol into its hemolymph to lower its freezing point (Crosthwaite et al., 2011). Overwintering compounds, such as glycerol or trehalose, can be synthesized from glycogen or lipids (Arrese and Soulages 2010). After winter, glycerol may be converted back into glycogen, but not at a one to one ratio (Chino 1960). Insects must also reach a minimum viable weight, where fat body lipid content is sufficient to initiate pupation and survive metamorphosis (Nijhout, 1975; Mirth & Riddiford, 2007).

Energy reserves are also important for flight of *A. planipennis* for mating and dispersal (Lelito et al. 2007, Wei et al. 2007). Glycogen is broken down into trehalose for flight muscle energy during short flights, while lipid becomes a flight energy source during prolonged flights (Beenakkers et al. 1984, Evenden et al. 2014). Because energy reserves have multiple purposes, a tradeoff may exist between energetic investments on overwintering and flight capacity. There is a tradeoff between flight capability and ovary development in *Gryllus firmus* Scudder (Orthoptera: Gryllidae), where long-winged flight morphs utilize stored lipid for flight fuel at the cost of developing smaller ovaries with lower reproductive ability (Zera, Sall, & Otto, 1999; Zera & Larsen, 2001). Likewise,

monarch butterflies have been found to expend large amounts of lipid while overwintering and must replenish energy reserves through feeding before large bouts of flight or reproduction (Chaplin & Wells, 1982). Other insects, such as *Manduca sexta* L. (Lepidoptera, Sphingidae), are unable to synthesize lipids as adults so must rely on lipid reserves obtained as larvae (Ziegler 1991). The role of adult feeding on energy reserves of *A. planipennis* is unknown.

Since 2002, the range of emerald ash borer has expanded to 30 states, with the current northwestern limit in eastern Minnesota (USDA, 2017). As emerald ash borer expands into regions with colder and more prolonged winters, a gradient may exist where *A. planipennis* can survive minimum winter temperatures, but experiences reduced fitness. The objectives of this study were to compare the energy reserves (defined as lipid, glycogen and free sugars) of *A. planipennis* that overwintered in two climactic regions of Minnesota with differing cold exposures and compare how adult feeding by both groups affects energy reserves and flight capacity. We hypothesize that colder winter conditions will increase mortality, and reduce energy reserves of surviving *A. planipennis*. Reduced energy reserves may ultimately reduce their flight dispersal capacity. We also hypothesize that adult *A. planipennis* feeding will increase energy reserves, and that increased feeding will increase flight capacity.

II. Methods

Study organisms

All *A. planipennis* were collected from naturally infested green ash logs that came from St. Paul or Minneapolis, MN. Logs were recovered from sites where sanitation for

management of *A. planipennis* was occurring. All logs were sourced from within a 6.5 km radius. Diameter at breast height (approximately 1.5 m above ground) of all trees used in these studies ranged from 8 - 30 cm.

Seeds of shamel ash, *Fraxinus uhdei* Wenzig, seeds were planted in 15 x 15 x 42 cm plastic pots (Treepot, Corvallis, OR) with mycorrhizae soil mix (Pro-Mix Mycorrhizae, Premier Horticulture INC, Quakertown, PA) in a greenhouse in January 2015. *Fraxinus uhdei* is an evergreen species that is capable of producing foliage year round and is commonly used to feed *A. planipennis* in captivity (Rodriguez-Saona et al. 2006, Crook et al. 2008, 2009). Trees were watered twice weekly and fertilized once weekly with 0.25 g of fertilizer composed of 20% by weight for N, P, and K (Plantex Corp., Brampton, Ontario Canada), throughout the study.

Effects of adult feeding on lipid reserves

In January 2015, ~12 green ash trees were cut into logs approximately 60 cm in length and left uncovered, outside our research facility in St. Paul, MN for the remainder of winter. In April 2015, before insect emergence, logs were retrieved from outdoors and randomly sorted into two batches. Logs of one batch were sorted placed in cardboard emergence tubes fitted with glass jars per Fahrner et al (2015). The remaining logs were placed into a walk-in cooler kept at 4°C to delay larval development until use. The first batch was replaced with the second after 28 days without any adult emergence. A total of 216 logs were used in 2015.

Feeding treatments: Emerging *A. planipennis* were sexed, weighed, and assigned a feeding treatment (0, 4, 8, 12, 16, or 20 d) and flight designation (no flight or 24 h on

flight mill) using a stratified random design at time of emergence. Individual *A. planipennis* were placed in 475 ml deli containers that were modified to have a 10 cm^2 hole covered with aluminum mesh for ventilation and a 7.62 cm length floral tube (Royal Imports, Brooklyn NY) inserted through the side to hold leaflets. Containers were kept on a lab bench at ~23° C and a photoperiod of 16:8 (L:D) h. Mature terminal leaflets of shamel ash were cut and rinsed with deionized water, and petiolules were placed in floral tubes to keep leaflets from desiccating during feeding trials. Beetles were allowed to feed *ad libitum* for the duration of their feeding treatment. Leaflets were replaced when ~50% of the leaf tissue was consumed or if leaves appeared desiccated. All leaves were imaged with a flatbed scanner after removal and surface area consumed was estimated using ImageJ software. Beetles not designated for flight treatments (below) were weighed and immediately frozen at -80° C until lipid analysis was performed.

Flight treatments: Custom computer-monitored flight mills were used to measure the flight capacity of adults, as described in Fahrner et al. (2014) and Kees et al. (2017). Flight tether arms were constructed using 20 cm long copper 108 American wire gauge (diameter ~0.171 mm), with a No. 1 insect pin serving as a central axis. After insects fed for a prescribed period, the pronotum was attached to one end of the tether arm using cyanoacrylate super glue (Loctite® Super Glue Gel; Henkel Corporation) (Machial et al. 2012). All beetles were checked by rotating the flight mill arm in an upright position by hand until elytra opened to ensure that the tether placement did not interfere with flight initiation. Beetles were placed on flight mills, and allowed to fly for 24h in continuous light (Fahrner et al. 2015). No food, water, or perches were provided. Each flight mill

was fitted with an infra-red (IR) sensor that recorded all sensitive movements of the tether arm. To account for rare but spurious movements of the tether arm, due to air currents or accidental bumps during assay initiation, bouts of flight were defined by the following parameters: flight speeds between 0.72 – 7.5 km h^{-1}, durations >5 s, and a minimum of three revolutions. After flight, beetles were removed from the flight mill arm by gently prying them from the dried glue. All beetles were reweighed and frozen at -80° C to preserve tissues until lipids were analyzed.

Lipid extraction using petroleum ether: Lipids were extracted with petroleum ether per McKee and Aukema (2015). Prior to lipid extraction, all beetles were placed in individual wells of a 24-well plate. Plates were placed in a drying oven set at 60° C for 24 h to desiccate beetles. Dry weights were recorded to the nearest 0.01 mg from all beetles within one hour of removal from the drying oven to minimize rehydration from ambient humidity. Lipid extraction was performed using 300 mL of petroleum ether circulating through an extraction column and condenser with a round-bottomed flask heated to 45° C. Individual beetles were placed in modified 0.5 ml micro-centrifuge tubes, allowing for petroleum ether to flow into and drain out of each tube during extraction. Paper labels with laser-printed numbers were inserted in each tube to identify beetles. Extractions ran with two flushes of the extractor column per hour for a total of 16 h. Beetles were again dried for 24 h at 60°C before being weighed a final time. Lipid weight was determined by subtracting the post-lipid-extraction dry weight from the initial dry weight. For each treatment combination, 8 - 25 beetles were tested for a total of 158 beetles in this study.

This experiment was repeated in 2016 with the following adjustments. Beetles were reared from a batch of 90 logs. In the previous experiment, we noted increased mortality in the 20 d treatment, likely due to a cessation of feeding sometime after 16 d, so feeding durations were limited to 0, 2, 4, 8, or 12 d. In addition, to account for starvation that occurs with the flight treatment, beetles that were not placed on the flight mill were not immediately frozen; rather, they remained in their container for 24 h, without access to food or water. For each treatment combination, between 8 and 15 beetles were tested for a total of 96 beetles in this study.

Effects of winter temperatures and feeding on energy reserves

In October 2015, green ash (*Fraxinus pennsylvanica*, *n*=30) that were naturally infested with *A. planipennis* were removed from St Paul, MN and brought to the University of Minnesota's St Paul campus. Each main stem was given an identification number before being cut into ~60 cm sections and labeled with stem number. Cut ends were sealed with paraffin wax to reduce desiccation. The logs were stored outside until use.

Two locations in Minnesota were selected to determine the effects of overwintering temperatures on energy reserves, nutrient levels, and flight capacity of *A. planipennis*: St. Paul, where below-bark temperatures were expected to be > -30° C, and Grand Rapids where below bark temperatures were expected to be < -30° C. Such low temperature extremes have been predicted to cause significant *A. planipennis* mortality (Venette and Abrahamson 2010, DeSantis et al. 2013).

On 8 January 2016, 70 logs were selected in a stratified random design, and placed onto two pallets of 35, with roughly equal numbers of logs from the same source trees for each group. One group was transported to Grand Rapids, where logs were secured with eyebolts and chains and kept in a gated, locked area, to comply with quarantine regulations. Logs were stored from 8 January – 3 March 2016, through the coldest part of winter. The other group was kept outside our research laboratory in St. Paul for the duration of the study. Both sets of logs were placed upright on wood pallets to keep them above the snowline. Hobo Pro v2, 2 ext temp recorders (Onset Computer Corp., Bourne, MA) were placed on the north and south faces of both pallets, with one probe inserted into the phloem of a log, to record below bark temperatures, the other probe inserted into a small white box to record ambient temperatures, for the duration of the experiment.

All logs were brought indoors on 8 March 2016 and placed in cardboard rearing tubes to allow adult beetles to emerge naturally. Adult beetles were collected daily from 14 April – 9 May 2016. All *A. planipennis* were sexed, weighed, and assigned a feeding (0, 4, 8, or 16 d) and flight treatment (no flight or 24 h on flight mill) using a stratified random design at time of emergence. For each treatment combination, energy reserves (specifically, lipid, glycogen, and sugars) were measured on between 11 – 23 beetles for a total of 134 beetles in this study. A sub-sample of logs from both overwintering locations were peeled 4 weeks after the last adult *A. planipennis* emerged to count the number of dead larvae, pupae, and adult *A. planipennis*. Mortality was measured by calculating the percentage of dead beetles from the total beetles recovered from each log.

Analysis of energy reserves using colorimetric assays: Prior to analysis, the elytra, wings, and legs were removed from each beetle to aid in processing. Analysis of energy reserves was performed on 8 individual beetles at a time. Each beetle was placed in a 1.5 ml microcentrifuge tube and vortexed with 1 ml of deionized water to rinse off external debris. The water was removed and beetles were crushed in 100 µl of sodium sulfate with a plastic pestle. A solution of chloroform-methanol (1:2 by volume, total volume 450 µl) was added to each vial, washing the pestle in the process. All vials were centrifuged at 1400 *g* for 5 minutes. Supernatant was removed and placed in a glass test tube (13mm x 100mm) for use in lipid and sugar assays. The precipitate was retained for analysis of glycogen.

Standard Curves: Known standards of lipid, glycogen, and sucrose were prepared as in Van Handel (1985a, 1985b). Lipid standards were prepared using soybean oil with concentrations of 0, 25, 50, and 100- 700 (in increments of 100) µg/ml and placed in clean test tubes. Glycogen standards were prepared with concentrations of 0, 1, 5, 10, 25, 50, 75, and 100 µg/ml, and stored in 1.5 ml micro-centrifuge vials until use. Sucrose standards were prepared with concentrations of 0, 1, 5, and 10- 50 (in increments of 10) µg/ml, and placed in clean glass test tubes. Fresh standards were made with each trial. Standards were processed as described below to produce a colorimetric change. Light absorbance was measured at predetermined frequencies (stated below) with a spectrophotometer (SpectraCount BS10000, Packard Instrument Company). Standard curves to relate the concentration of each compound to absorbance were created using linear or second order polynomial regressions

Lipid Assay: Lipids were analyzed using methods modified from Van Handel 1985. From each standard or supernatant, 200 µl was transferred to a clean test tube and heated to 90° C until all liquid evaporated. Each tube was cooled to ambient temperature and 200 µl of sulfuric acid was added. The samples and standards were reheated to 90° C for 10 minutes. Test tubes were cooled to ~20° C and filled to a total of 5 ml with vanillin reagent. The solution was allowed to react for 10 minutes before being plated. Two 200 µl aliquots from each sample and standard were placed into wells of a 96-well microwell plate. Absorbance at 490 nm was measured. The absorbance readings for the two replicates were averaged to estimate the lipid content. The final measurement of lipid was multiplied by 2.75 µl to account for dilution throughout the assay, yielding a total mass estimate in µg.

Glycogen Assay: Glycogen was analyzed per Olson et al. (2000). To each microcentrifuge tube containing the precipitate, 1 ml of anthrone reagent (see Olson et al., 2000) was added. Tubes were vortexed for ~30 seconds to dissolve the precipitate and centrifuged at 1200 g for two minutes to separate out remaining solids. Half of this solution (500 µl) was transferred to a new tube, along with an additional 500 µl of anthrone reagent. Standards were filled to a total of 1 ml with anthrone reagent. The samples and standards were heated at 90° C for eight minutes. Two 200 µl aliquots of each sample and standard were placed into wells of a 96-well microwell plate. Absorbance at 620 nm was measured. The absorbance readings for the two replicates were averaged to estimate the lipid content. The final measurement of glycogen was

multiplied by 2.0 µl to account for dilution throughout the assay, yielding a total mass estimate in µg.

Sugars Assay: We followed the procedures of Olsen et al. (2000) to measure concentrations of disaccharides using a hot anthrone method. A 100 µl aliquant of supernatant was transferred to a clean test tube (as previously described) and heated for 4.5 minutes. Test tubes were placed on ice to cool before 750 µl of anthrone reagent was added. Vials containing standards were filled to a total of 1ml with anthrone reagent. The samples and standards were heated at 90° C for 12 minutes. Two replicates of 200 µl solution from each sample and standard were placed into wells of a 96-well microwell plate. Absorbance at 620 nm was measured. The absorbance readings for the two replicates were averaged to estimate the disaccharide content. The final measurement of sugars was multiplied by 5.5 µl to account for dilution throughout the assay, yielding a total mass estimate in µg.

Statistics

All statistical analyses were conducted using R software (ver.3.3.1). Linear regression models were used to relate leaf area consumption to adult age to estimate feeding rate. Linear regression was used to relate leaf area consumption to change in mass and lipid reserves. Only non-flown beetles were tested for the effects of feeding on lipid reserves to prevent confounding effects of flight. Lipid was expressed as percent of fresh mass, since a strong correlation existed between lipid mass and fresh weight. Linear regression was used to relate beetle mass to flight distance. Analysis of variance

(ANOVA) was used to compare the effects of adult age and sex on flight metrics (i.e. number of bouts, distance, flight time, and velocity). A two-sample *t*-test was performed to compare lipid reserves of flown vs non-flown beetles.

To test for differences in low temperatures, a dataset of the difference of daily low temperatures was constructed and tested for autocorrelation using Durbin-Watson tests. After data was thinned (by removing every other day) to remove temporal autocorrelation that can bias inferential tests, a one sample *t*-test was performed to compare the mean temperature differences between the two sites (at α=0.05). An ANOVA was used to compare mortality between sites. Linear regressions were used to relate leaf area consumption with lipid, glycogen, and sugar reserves. ANOVAs were performed to compare glycogen and sugar reserves of flown vs non-flown beetles. Linear regression was used to relate glycogen content to total flight distance. In all regressions, non-significant terms ($P \geq 0.05$) were iteratively removed by using a backwards elimination procedure. Final models were selected based on model fit (e.g. adjusted R^2 values) and parsimony.

III. Results

Feeding over time: In 2015, beetles consumed leaf tissue at a rate of 1.42 cm^2/d ($F_{1,137}$= 413.9, $P< 0.001$). Beetles would occasionally consume over 20 cm^2 after a week of feeding. The feeding rate was similar in 2016 (Fig. 1: A, B). The mass of males that fed exhibited 5% higher body mass increases than females, irrespective of adult age in 2015 ($F_{1,123}$= 9.18, P= 0.003). However, there were no significant differences in mass change between sexes in 2016. Body mass changed with adult age, with an initial mass gain in

the first 8 days, followed by a slight decline in mass (Fig. 1: C, D). However, these patterns were not significantly correlated with leaf area consumption ($F_{1,137}$= 0.527, P= 0.469).

***Effects of feeding on lipid*:** Beetle lipid reserves were not affected by leaf area consumption ($F_{1,67}$= 0.017, P= 0.879) in 2015 or in 2016. (Fig. 2). Lipid (% of fresh mass) did not change significantly with adult age ($F_{1,95}$= 1.775, P= 0.186). These results were similar in 2016. The mean lipid content for beetles was 2.32% (± 0.16%) in 2015, and 1.66% (±0.14%) in 2016.

Flight tests: Flight distance increased significantly with the age of adult beetles in 2015, with total flight distance increasing by 47 m/d (Fig. 3-A). A similar result was obtained in 2016 with flight distance increasing by 91 m/d (Fig. 3-B). The maximum distance flown was 5.6 km in 2015, and 3.5 km in 2016. Table 1 shows flight performance metrics of beetles, based on adult age and sex, in 2015 and 2016. No significant differences between sexes were found for either year. Velocity increased with adult age in 2015, but this trend was not apparent in 2016.

Effects of winter temperatures and feeding on energy reserves

Winter temperatures and mortality: Below bark temperatures experienced by *A. planipennis* larvae were significantly colder in Grand Rapids, MN than in St Paul, MN ($F_{1,27}$= 62.09, P< 0.0001). Temperatures dropped below -30° C four times in Grand Rapids, while temperatures in St Paul remained above -30° C. The lowest below bark temperatures recorded for the Grand Rapids and St Paul were -34° C and -26.3° C,

respectively (Fig. 5). Overwintering mortality was significantly greater in Grand Rapids, about 50%, compared to St. Paul, which was approximately 20% ($F_{1,8}$= 28.84, P= 0.0006, n=10). Totals of 34 and 90 adults were collected from logs from Grand Rapids and St Paul, respectively. However, overwintering location had no significant impacts on emergence mass, feeding rate, nutrient reserves, or flight performance. These non-significant differences are not reported.

***Effects of feeding on energy reserves*:** Beetle lipid reserves were not affected by leaf area consumption ($F_{1,67}$= 0.017, P= 0.879) (Fig. 5-A). The average lipid content was 3.13% (± 0.13%). Glycogen content increased at a rate of 0.4 µg/cm^2 leaf area consumed, although this trend was only marginally significant ($F_{1,67}$= 3.265, P= 0.075) (Fig. 5-B), while total sugars significantly increased with leaf area consumption ($F_{1,67}$= 7.815, P= 0.007), at a rate of 2.7 µg/cm^2 (Fig.5-C).

Effects of flight on energy reserves: Beetle mass at the time of flight was a strong predictor of flight distance, along with adult age ($F_{2,55}$= 11.48, P< 0.0001). Heavier and older beetles that were allowed to feed *ad libitum* flew farther on average. Both glycogen ($F_{1,122}$= 4.41, P= 0.039) and sugar ($F_{1,109}$= -2.10, P= 0.038) content (in mg) were significantly lower in flown vs non-flown beetles, with flown beetles having 0.01 mg less glycogen and 0.04 mg less sugars. Glycogen percent (of fresh mass) decreased with increasing flight distances at a rate of 0.024%/km ($F_{1,53}$= 5.45, P= 0.023) (Fig. 6). Table 2 shows flight performance metrics of beetles, based on adult age and sex. No significant differences in flight metrics were found between sexes.

General flight observations: Across all studies, 143 out of the 165 *A. planipennis* that were placed on flight mills initiated flight. Only 71% of unfed individuals initiated flight at least once during 24 h flight trials, while 93% of fed individuals initiated flight at least once, a difference that was significant (Z= 3.56, P< 0.001, n= 155) (Fig. 7). Adults flew an average of 0.54± 0.08 km (range: 0.002 - 5.58 km) within 24 h under constant light.

IV. Discussion

Our findings suggest that few sub-lethal effects exist between *Agrilus planipennis* overwintering in Grand Rapids, MN and St. Paul, MN. Although mortality was significantly higher among *A. planipennis* larvae that had overwintered in Grand Rapids, MN than in St. Paul, MN, feeding and flight performance among emerged adults was not different. As such, *A. planipennis* do not appear to be utilizing more energy reserves in colder locations. One potential reason for similar energy reserve levels for both groups could be that the temperature threshold for energy reserve metabolization in *A. planipenni*s is relatively high. Hayakawa and Chino (1981) found a temperature dependent relationship for glycogen conversion into overwintering compounds in pupae of *Samia cynthia* Drury (Lepidoptera: Saturniidae), where glycogen is rapidly converted into trehalose once exposed to low temperatures. Furthermore, the production of trehalose was initiated through enzymes that activate at 2° C, and maintain at colder temperatures (Hayakawa and Chino 1982). *Agrilus planipennis* may have a similar response, where mechanisms for cold tolerance activate in relatively warm temperatures and do not differ at colder temperatures.

All *A. planipennis* collected had greater masses of lipid reserves than glycogen or sugars. This may indicate that lipid is preserved through winter, while glycogen and sugars are used for production of overwintering compounds. In this study, adult feeding did not have any impact on lipid reserves. This may indicate that *A. planipennis* is unable to synthesize lipids as adults. Similarly, lipid content did not significantly decrease with flight, suggesting that lipids may be more important for purposes other than flight fuel, such as reproduction. Lipid is often important in ovary development or spermatophore production (Briegel, 1990; a. J. Zera & Larsen, 2001; Rutledge & Keena, 2012).

Although we were unable to detect significant differences in energy reserves between overwintering locations, there are likely differences in energetic demands during overwintering at different temperatures. Although not significant, a higher lipid content in *A. planipennis* overwintered in Grand Rapids versus St. Paul may be due to a lower metabolic rate in colder temperatures. Many insects induce a depression in metabolic rates during quiescence. Warming temperatures then increase metabolism at the end of quiescence (Furusawa et al. 1982, Liquido and Irwin 1986, Storey and Storey 2012). Beetles in St Paul experienced warmer temperatures more often, and may have expended more lipid to fuel a higher metabolic rate. Lipid is commonly released into hemolymph during periods of starvation, and larvae overwintering in pupal chambers stop feeding (Ziegler, 1991; Arrese & Soulages, 2010).

It is interesting to note that the mean glycogen level was nearly identical for both groups of beetles. If *A. planipennis* invest in antifreeze compounds only at the onset of winter, similar glycogen levels between groups would be expected, as all beetles in this

study originated from the same population and began overwintering in St Paul, MN. Crosthwaite et al., (2011) found that *A. planipennis* produce large amounts of glycerol in November, and levels remain stable from December- March. It has also been found that *A. planipennis* begins to catabolize glycerol at the onset of warmer temperatures, and that this process is irreversible (Sobek-Swant et. al, 2012). It seems unlikely that *A. planipennis* is capable of increasing hemolymph glycerol content in mid-winter, or augment glycerol levels when exposed to ambient temperatures.

This experiment did not incorporate the difference of winter duration between the two locations. Northern regions generally have longer durations of colder temperatures, which may create a higher demand for overwintering compounds. Both temperature and time are important factors affecting fitness after overwintering. Some parasitoid wasps have been found to have strong reduction of fitness with increasing duration of exposure to cold temperatures (Ellers and Van Alphen 2002). The duration of temperatures below 0° C is much longer in northern Minnesota than the southern portion of the state that *A. planipennis* infests (Minnesota DNR). These prolonged exposures in northern regions could increase mortality or lower energy reserves in *A. planipennis*.

Beetles in this study typically did not fly over 0.5 km in 24 h. These distances are somewhat shorter than what has been previously reported for *A. planipennis* flight (Taylor et al. 2010, Fahrner et al. 2015), where reported mean flight distances for *A. planipennis* originating in Michigan were greater than 1 km. To our knowledge, this is the first *A. planipennis* flight study using a Minnesota population. These shorter flight distances may be a result of the colder climate in Minnesota. Beetle mass at the time of

flight was a strong predictor of flight distance. Minnesota populations of *A. planipennis* may have smaller individuals on average due to a shorter growing season, where larvae have less time to gain mass. Body size has previously been correlated with season length, with regions of shorter growing seasons having smaller sized insects (Mousseau and Roff 1989). Higher latitudes are also associated with smaller individuals in some insect species (Hawkins 1995).

Nearly all *A. planipennis* adults that were allowed to feed on leaf tissue ate vigorously. However, feeding did not significantly affect mass or lipid content. Although feeding did significantly increase sugars, and to a lesser extent, glycogen, the increases were slight and unlikely to substantially increase flight potential. It appears more likely that adult feeding serves to maintain water content. It was incidentally noticed that beetles that did not feed would desiccate and die within 2 days.

Although we did not detect any sub-lethal effects of cold with respect to dispersal capacity, potential impacts may exist. Due to increased mortality in Grand Rapids, MN, our sample size may have been too small to detect significant differences in energy reserves or flight capacity between overwintering locations. There is also the possibility that our treatment locations were not different enough to illicit sub-lethal effects. Still, other potential trade-offs may exist. We did not test for any fecundity differences. Tradeoffs have been noted between energy expenditure during overwintering and fecundity as adults in goldenrod gall flies, for example (Irwin and Lee, 2000). Lipid reserves are also important for successful egg development in many insects (Anderbrant 1988, Zera and Larsen 2001, Sisterson et al. 2015).

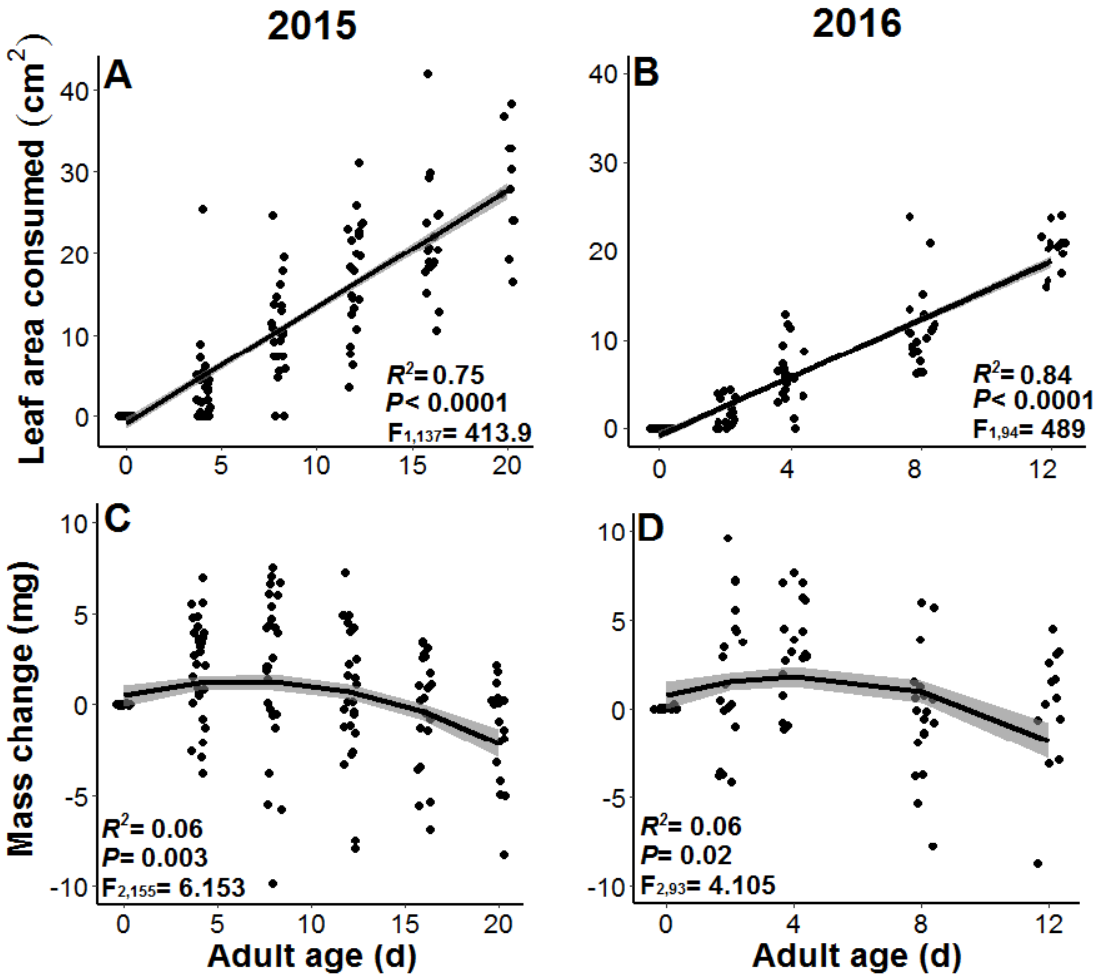

Figure 1. Relationship of leaf area consumed (A, B) and mass change (C, D) of *Agrilus planipennis* to adult age in 2015 (left) and 2016 (right). Gray ribbons associated with regression lines represent standard errors.

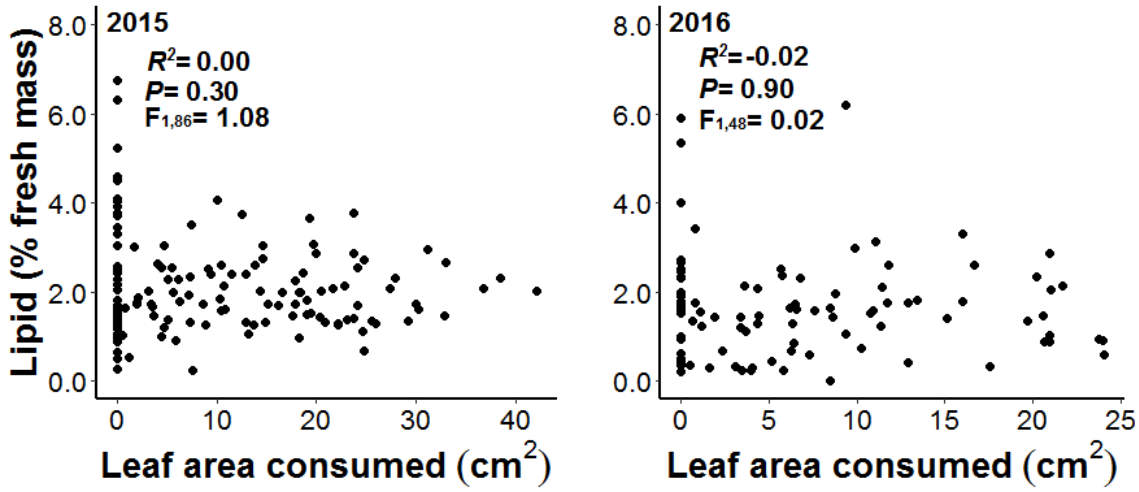

Figure 2. Lipid content vs leaf area consumed by *Agrilus planipennis* adults in 2015 (left) and 2016 (right). Grey ribbons associated with regression lines represent standard errors.

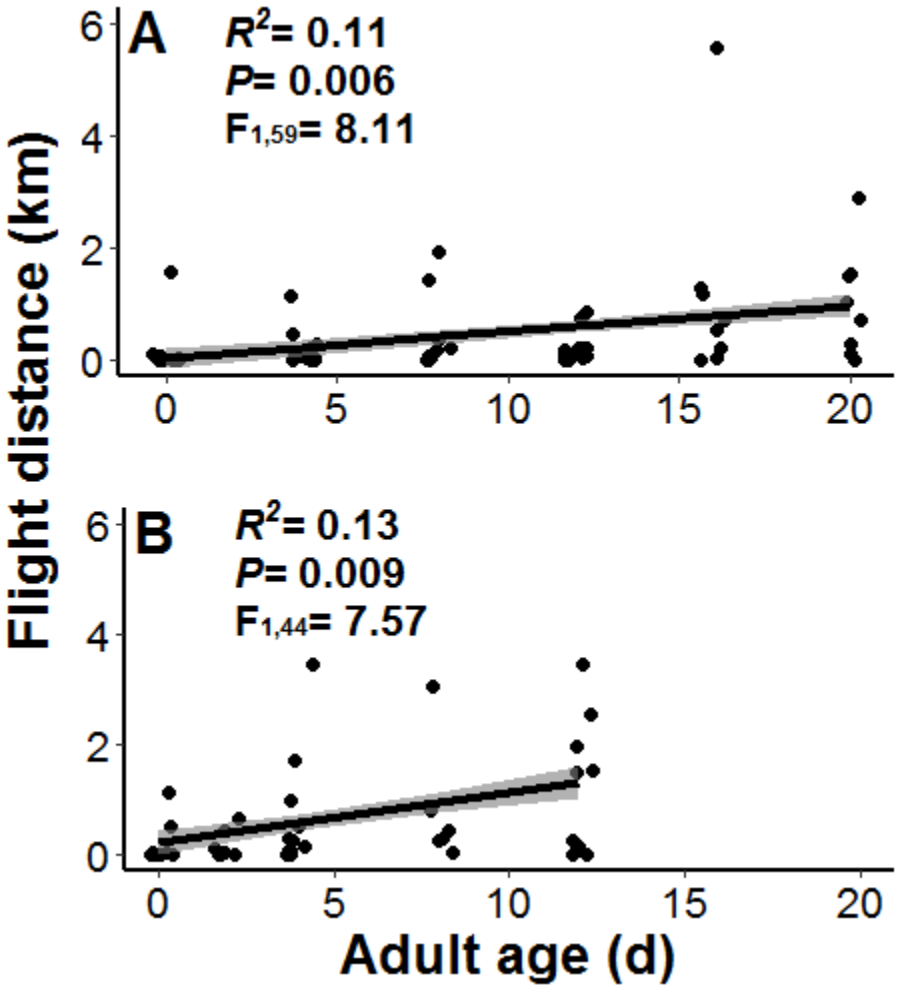

Figure 3. Total flight distance of *Agrilus planipennis* after 24 h on a flight mill in 2015 (A) and 2016 (B), vs adult age of *Agrilus planipennis*. Gray ribbons associated with regression lines represent standard errors.

Table 1: Mean flight metrics (±SE) of *Agrilus planipennis* by adult age and sex on flight mills in 2015 and 2016, and associated ANOVA results. If significant differences among ages were present, letters beside values of females denote similar values. No significant differences were found between sexes.

2015	Bouts		Distance (km)		Time (m)		Velocity (kph)		n	
Adult Age	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**
2015 0	10.2 ± 1.4	1.0 ± 0.0	0.354 ± 0.302	0.003 ± 0.002	9.6 ± 7.7	0.1 ± 0.1	1.50 ± 0.08**a**	1.81 ± 0.91	5	2
4	41.8 ± 17.6	5.5 ± 1.5	0.364 ± 0.166	0.056 ± 0.017	10.0 ± 4.1	1.4 ± 0.3	1.46 ± 0.08**a**	1.72 ± 0.32	6	4
8	28.8 ± 7.7	17.0 ± 0.0	0.544 ± 0.352	0.745 ± 0.695	12.4± 6.6	18.4 ± 15.7	1.89 ± 0.44**ab**	1.32 ± 0.30	4	2
12	22.6 ± 7.5	16.5 ± 6.5	0.244 ± 0.086	0.064 ± 0.006	5.9 ± 1.5	17.4 ± 15.4	1.88 ± 0.22**ab**	1.49 ± 0.01	11	2
16	39.3 ± 30.1	9.5 ± 1.5	1.500 ± 0.841	0.269 ± 0.266	28.7 ± 20.3	5.6 ± 4.2	2.18 ± 0.22**ab**	1.52 ± 0.36	6	2
20	30.5 ± 11.4	46.0 ± 40.0	0.860 ± 0.247	1.457 ± 1.443	13.5 ± 5.0	31.5 ± 30.8	2.52 ± 0.40**c**	1.83 ± 0.02	6	2
	$F_{6,54}$= 1.22	*P*= 0.313	$F_{6,54}$= 2.37	*P*= 0.041	$F_{6,54}$= 1.11	*P*= 0.372	***F*$_{6,54}$= 3.98**	***P*= 0.002**		
2016 0	12.3 ± 10.3	11.5 ± 8.5	0.202 ± 0.164	0.292 ± 0.282	4.1 ± 3.3	4.9 ± 4.3	2.67 ± 0.36	2.32 ± 0.62	3	4
2	63.0 ± 54.0	6.3 ± 2.3	0.242 ± 0.209	0.211 ± 0.153	11.3 ± 9.8	3.7 ± 2.7	1.28 ± 0.05	2.36 ± 0.41	2	4
4	45.5 ± 9.4	30.5 ± 8.6	1.095 ± 0.542	1.089 ± 0.797	23.5 ± 10.3	20.9 ± 15.7	2.14 ± 0.25	2.42 ± 0.27	6	4
8	74.7 ± 53.7	13.3 ± 7.4	1.255 ± 0.898	0.377 ± 0.221	30.3 ± 23.0	8.8 ± 5.8	2.42 ± 0.16	2.15 ± 0.21	3	3
12	38.6 ± 15.8	29.0 ± 22.0	1.112 ± 0.386	1.810 ± 1.658	18.7 ± 8.0	35.0 ± 32.6	2.54 ± 0.22	3.21 ± 0.39	7	2
	$F_{5,40}$= 2.14	*P*= 0.082	$F_{5,40}$= 1.83	*P*= 0.128	$F_{5,40}$= 1.49	*P*= 0.214	$F_{5,40}$= 1.58	*P*= 0.188		

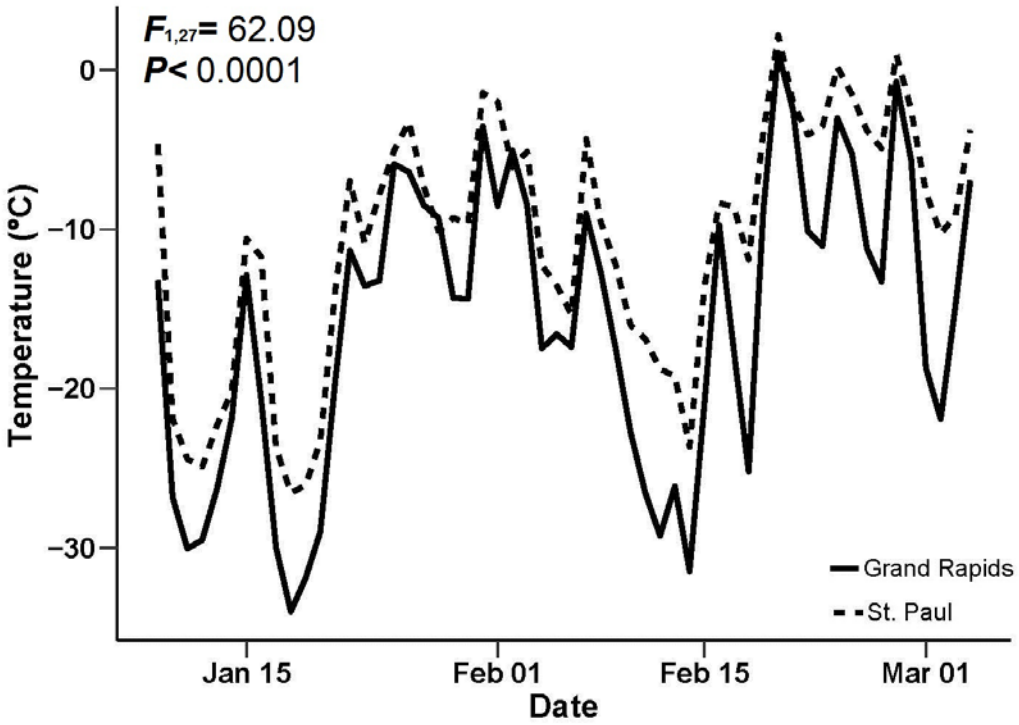

Figure 4. Recorded below- bark low temperatures of *Fraxinus pennsylvanica* logs stored in either Grand Rapids, MN or St. Paul, MN from 8 January 2016 to 3 March 2016.

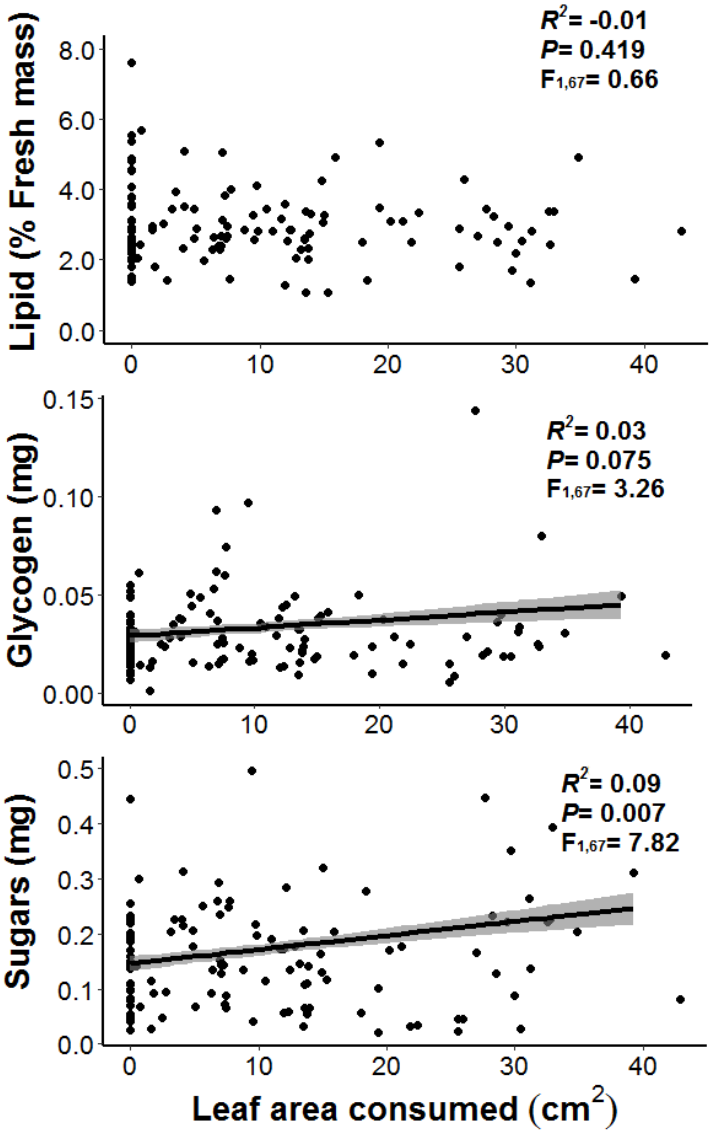

Figure 5. Lipid (top), glycogen (middle), and sugars (bottom) of adult *Agrilus planipennis* vs. leaf area consumed. Gray ribbons associated with regression lines represent standard errors.

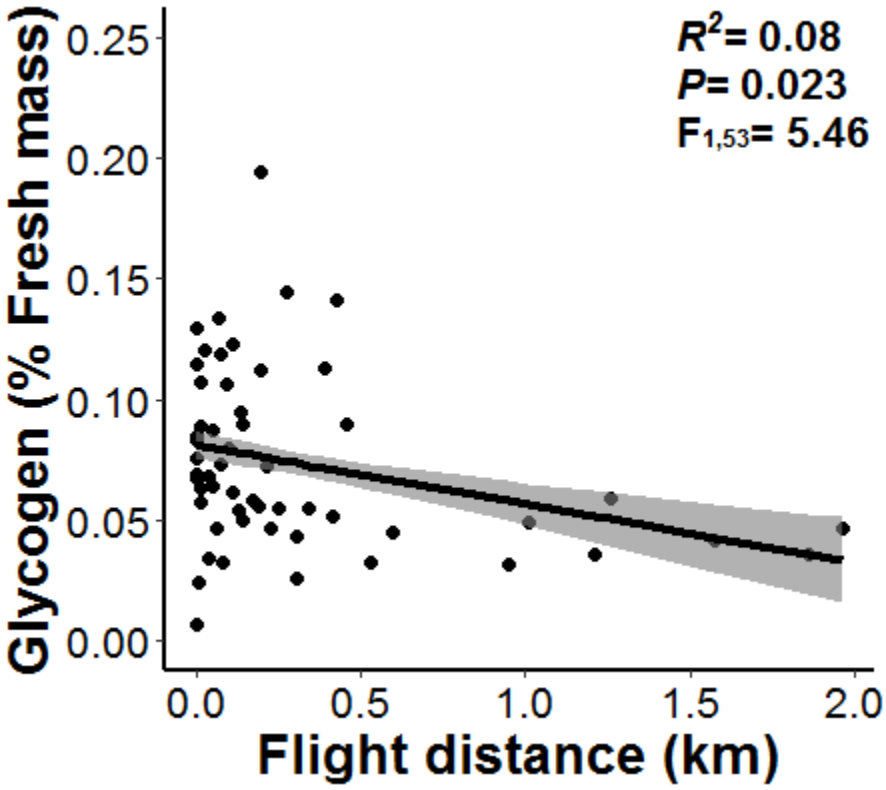

Figure 6. *Agrilus planipennis* glycogen content (% of fresh mass) vs. total distance flown (km) in 24 h on flight mill in 2016.

Table 2: Mean flight metrics (±SE) of *Agrilus planipennis* by adult age and sex on flight mills in 2016 with associated ANOVA results. If significant differences among ages were present, letters beside values of females denote similar values. No significant differences were found between sexes.

	Bouts		Distance		Time		Velocity		n	
Adult Age	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**
0	21.2 ± 11.0	5.8 ± 2.4	0.177 ± 0.052**a**	0.133 ± 0.065	5.0 ± 2.0**a**	2.8 ± 0.9	2.46 ± 0.31	1.92 ± 0.41	9	4
4	29.3 ± 17.3	39.8 ± 12.3	0.255 ± 0.147**a**	0.172 ± 0.050	6.8 ± 3.2**ab**	7.4 ± 2.1	1.94 ± 0.20	1.33 ± 0.07	6	6
8	22.2 ± 5.6	20.7 ± 8.0	0.522 ± 0.243**ab**	0.170 ± 0.066	10.5 ± 4.2**ab**	4.1 ± 1.4	2.09 ± 0.20	2.05 ± 0.28	9	6
16	26.5 ± 10.9	23.7 ± 8.8	0.793 ± 0.288**b**	0.513 ± 0.206	15.3 ± 5.4**b**	10.8 ± 4.1	2.59 ± 0.42	2.26 ± 0.35	6	5
	$F_{4,53}$= 1.33	*P*= 0.271	$\mathbf{F_{4,53}= 4.09}$	***P*= 0.006**	$\mathbf{F_{4,53}= 3.32}$	***P*= 0.017**	$F_{4,53}$= 2.68	*P*= 0.041		

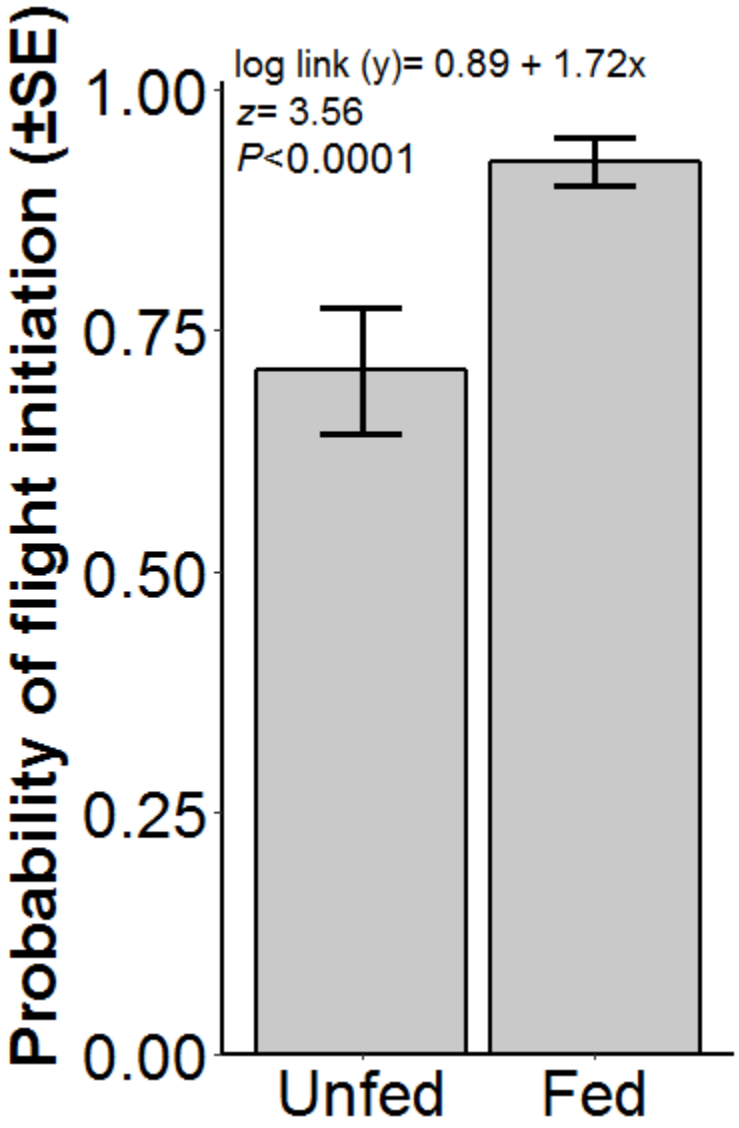

Figure 7. Comparison of flight initiation between unfed (*n*= 48) and fed (*n*= 107) *Agrilus planipennis* (±SE).

Thesis Conclusions

1) Increased mortality is the only significant difference between overwintering locations. Despite a mortality rate twice as high as observed in St. Paul, MN, *A. planipennis* that survived had similar levels of lipid, glycogen, and sugars. No differences in feeding rate or flight capacity were observed.

2) Adult feeding does not increase *Agrilus planipennis* lipid reserves. Lipid reserves in *A. planipennis* are nearly 2.5% of fresh mass, and do not change with leaf consumption or adult age. Adult *A. planipennis* do not appear to synthesize lipids.

3) Adult feeding by *A. planipennis* increases glycogen and sugars. Both glycogen and sugars increase with leaf area consumption. However, these increases are slight and not likely to increase dispersal capacity.

4) Glycogen appears to be fuel source for *A. planipennis* flight. Glycogen content decreases with increasing flight distance. Although feeding increases glycogen slightly, dispersal capacity is primarily determined by beetle age and mass**.**

Appendix 1
Lipid and water content of overwintering *Agrilus planipennis* larvae across development stages and overwintering locations

I. Introduction

Emerald ash borer (*Agrilus planipennis* Fairmaire) is a severe pest of North American ash species (*Fraxinus* spp.). *A. planipennis* larvae overwinter in their host tree, either as late instars in pupal chambers within the outer sapwood, or as earlier instar larvae still in feeding galleries (Cappaert et al. 2005, Poland and McCullough 2006, Wei et al. 2007). In order to survive winter temperatures, larvae of many insects undergo physiological changes to increase cold tolerance (Sinclair et al. 2003, Crosthwaite et al. 2011, Storey and Storey 2012).

Minnesota is the northwestern edge limit of *A. planipennis* in the United States, where winter temperatures potentially drop low enough to cause significant mortality for this species (Venette and Abrahamson 2010, DeSantis et al. 2013). This study measured lipid and water content of overwintering *A. planipennis* larvae from Minnesota and Ohio. We hypothesize that lipid content will increase with larval development, and that water content will be reduced in pre-pupal larvae. We also hypothesize that lipid content will be higher in larvae that overwintered in Ohio.

II. Methods

In Jaunuary 2015, *A. planipennis* larvae were extracted from green ash (*Fraxinus pennsylvanica*) logs collected in Ramsey County, Minnesota. Larvae were sorted into three categories: early, defined as any larva, typically 4th instar, that was not in a pupal

chamber; J-stage, defined as 4th instar larvae in a pupal chamber; and pre-pupae, defined as late 4th instars inside pupal chambers with contracted bodies (commonly described as a "fat head"). Each larva was stored in a micro-centrifuge vial and frozen at -80° C until lipid extraction was performed.

In April 2016, green ash logs originating from either Minnesota or Ohio were collected and overwintered in either of the two states. Late instar larvae collected from pupal chambers, frozen at -80° C, and stored in micro-centrifuge vials until lipid extraction was performed.

All larvae were placed in individual wells of a 24-well plate and in a drying oven set at 60° C for 24 h to remove all water from the beetles. Dry weight of each larva was recorded to the nearest 0.01 mg within one hour of removal from the drying oven to minimize rehydration from ambient humidity. Total water content was calculated by subtracting larval dry weight from fresh mass. Percent water was calculated by dividing the mass of water by the fresh mass of the larva.

Lipid extraction was performed using 300 mL of petroleum ether circulating through an extraction column and condenser with a round-bottomed flask heated to 45º C. Larvae were individually placed in modified 0.5 ml micro-centrifuge vials that allowed petroleum ether to flow into and drain out of each tube during extraction. Paper labels with laser-printed numbers were inserted in each tube to identify beetles. Extractions ran with two flushes of the extractor column per hour for a total of 16 h. Larvae were again dried for 24 h at 60° C before being weighed a final time. Lipid weight

was determined by subtracting the post-lipid-extraction dry weight from the initial dry weight.

III. Results and Discussion

Water content (% of fresh mass) was lowest in fat-head larvae (61.6%± 1.0%), while early instar larvae possessed the highest water content (65.5%± 1.1%); however, this trend was not significant ($F_{2,21}$= 2.34, P= 0.12). Lipid content (% of fresh mass) increased with larval development (Fig. A1-1), although this trend was not significant ($F_{2,21}$= 1.91, P= 0.17).

There were no distinguishable patterns in regards to larval origin or overwintering location. Lipid content was lowest in larvae that originated and overwintered in Minnesota, but highest in larvae that originated in Minnesota but overwintered in Ohio ($F_{3,44}$= 0.53, P= 0.66) (Fig. A1-2).

A reduction in water content prior to overwintering is a common adaptation to increase cold tolerance in insects (Bale 2002, Sinclair et al. 2003). Numerous species of insects also are known to accumulate lipids prior to overwintering (Pullin et al. 1987). Interestingly, larvae collected in 2016 had much higher lipid percentage of fresh mass than larvae collected in 2015. Since larvae were processed three months later in 2016, it is possible that post-winter pre-pupae synthesize lipids from other energy reserves. Aside from being an energy reserve, lipids also produce large amounts of water upon oxidation, which can be an important source of water after overwintering (Downer and Matthews 1976). Lipid is also an important energy source during metamorphosis in many insects.

Agrilus planipennis pupates soon after overwintering, without feeding between these events, so lipid accumulation as mature late instar larvae could be important, as both energy and water sources, for survival to adulthood.

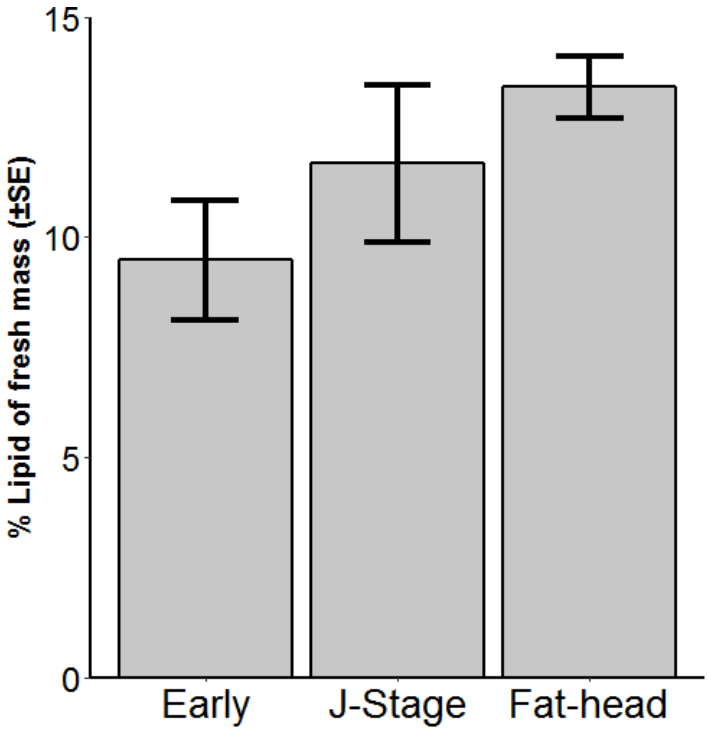

Figure A1-1. Mean percent lipid of fresh mass for different larval stages of *Agrilus planipennis* collected from Minnesota in 2015. Bars represent standard errors.

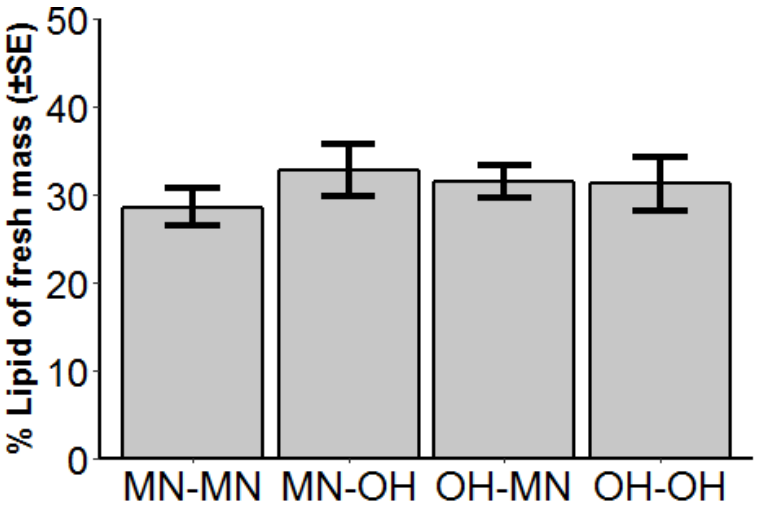

Figure A1-2. Mean percent lipid of fresh mass of *Agrilus planipennis* pre-pupae with state of origin and overwintering location in 2016. Bars represent standard errors.

Appendix 2:

Comparison nitrogen and carbon contents of lab grown *Fraxinus uhdei* to Minnesota populations of *Fraxinus nigra* and *Fraxinus pennsylvanica*

I. Introduction

Insect herbivores rely on their host plant as a source of protein and carbohydrates (Chapman 1998, Behmer 2009). Even insects that specialize on one or a few host plant species still experience variation in the concentrations of proteins and carbohydrates that are consumed, due to genetic and environmental differences among hosts (Despland 2006, Behmer 2009). Differences in diet quality can affect the performance of insects, in either growth rate, flight, or fecundity (Waldbauer et al. 1984, Despland 2006, Lee et al. 2006).

Numerous studies on emerald ash borer (*Agrilus planipennis* Fairmaire) use greenhouse grown *Fraxinus uhdei* Wenzig as a food source for adult beetles in experiments (Rodriguez-Saona et al. 2006, Crook et al. 2009, Taylor et al. 2010). *Fraxinus uhdei* is a commonly used, as it is an evergreen *Fraxinus* species that grows easily in greenhouse conditions. As many of these studies attempt to approximate the natural behavior and performance of *A. planipennis*, it is important to determine if the quality of hosts grown in a greenhouse are comparable to what is available to the beetles in the wild.

In this experiment, we compared the carbon and nitrogen contents of leaf tissue in lab grown *F. uhdei* to populations of *Fraxinus pennsylvanica* (Marshall) and *F. nigra* (Marshall) from eastern Minnesota. These elements were used as they are related to

overall protein and carbohydrate contents (Loomis 1997). We hypothesize that nitrogen content will be greater in lab grown *F. uhdei* than in *F. pennsylvanica* or *F. nigra,* but expect no differences in carbon content.

II. Methods

In January 2015, *F. uhdei* seeds were planted in 15 x 15 x 42 cm plastic pots (Treepot, Corvallis, OR) with mycorrhizae soil mix (Pro-Mix Mycorrhizae, Premier Horticulture INC, Quakertown, PA). Trees were watered twice weekly and fertilized once weekly with 0.25 g of fertilizer composed of 20% by weight for N, P, and K (Plantex Corp., Brampton, Ontario, Canada), throughout the study.

In July 2015, four locations with populations of *F. pennsylvanica* and *F. nigra* in eastern Minnesota were selected. There was one location in each of the following counties: Anoka, Carver, Mille Lacs, and Winona (Table A2-1). At each location, five apparently healthy trees of each species were randomly selected for leaf collection. However, in Winona County, only two *F. nigra* were sampled, as the majority of ash trees in the area were in decline from *A. planipennis* infestation. All trees were at least 5 m apart. The diameter at breast height (DBH) was measured for each tree. From each tree, samples consisting of 10 terminal leaflets were collected radially around the lower canopy, placed in plastic bags and stored in a cooler for transport. Similarly, ten terminal leaflets were collected from five *F. uhdei,* randomly selected in the greenhouse. DBH was not collected for these seedlings, as all trees were less than 3 cm in diameter. All leaflets were placed in a drying oven set at 60° C for 24 h. Dried leaves were stored in sealed plastic bags prior to analysis.

Before analysis, samples were ground into a fine powder (~0.7 cm^2) using a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA). Samples were then sent to the Research Analytical Laboratory at the University of Minnesota (St. Paul, MN) where nitrogen and carbon percentages (of dry weight) were determined through a combustion method using a LECO FP-528 Nitrogen Analyzer (Simonne et al. 1994, Matejovic 1995). A carbon-nitrogen (C:N) ratio was calculated by dividing the percent of carbon by the percent of nitrogen.

This study was repeated in July 2016 with the exception that the sites in Mille Lacs and Winona counties were not sampled.

Statistics

A variance components analysis was performed with leaf sample data to compare the relative tree variation with site variation in C:N ratio. Variation attributed to tree and variation among sites was measured from the standard deviations of the respective random effects in linear models fitting the C:N ratio to an intercept. The standard deviations of each component were squared to obtain the relative variances attributable to inter-tree and inter-site effects. Analysis of variance (ANOVA) was used to compare the effects of species on C:N ratios.

III. Results and Discussion

Variation in C:N ratio was greater among sites than among trees within a site. Inter-site variance was estimated to be 5.9, while the estimated variance among trees within a site was 0.08. Results were similar in 2016. After accounting for site and tree

variations, *F. pennsylvanica* was found to have a significantly higher C:N ratio than either *F. nigra*, or *F. uhdei* ($F_{2,19}$= 3.58, P=0.0479, Fig. A2-1). There was no significant difference in leaf nitrogen in 2015 ($F_{2,19}$= 2.34, P=0.124). This was also true for 2016. A positive correlation was found between DBH and leaf carbon content in *F. pennsylvanica*, however, this was not found in *F. nigra* (Fig. A2-2). This trend was similar in 2016.

These results show that lab grown *F. uhdei* are acceptable facsimiles for native populations of *Fraxinus* spp. in Minnesota in terms of nitrogen and carbon content. Despite weekly fertilization of the lab grown *F. uhdei*, there were no significant differences in CN ratios between *F. uhdei* and *F. nigra*, and all 3 species had similar levels of nitrogen.

Table A2-1. Coordinates of Sample sites for *Fraxinus nigra* and *Fraxinus pennsylvanica* in 2015 and 2016.

County	Latitude (°N)	Longitude (°W)
Anoka	45.2880	-93.1389
Carver	44.5864	-93.6242
Mille Lacs	45.9122	-93.5651
Winona	43.9492	-91.3893

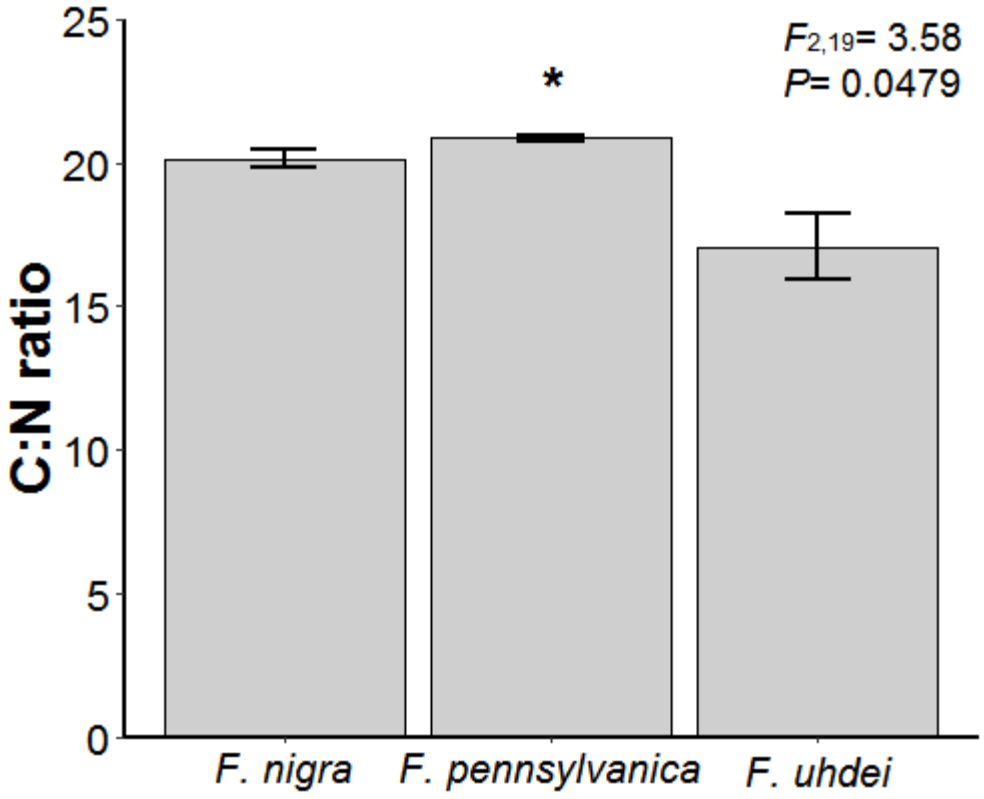

Figure A2-1. Mean carbon to nitrogen ratios of *Fraxinus nigra*, *F. pennsylvanica*, and *F. uhdei* in July 2015. Bars represent standard deviation after accounting for variance attributed to location and tree. Asterisk denotes a significant difference.

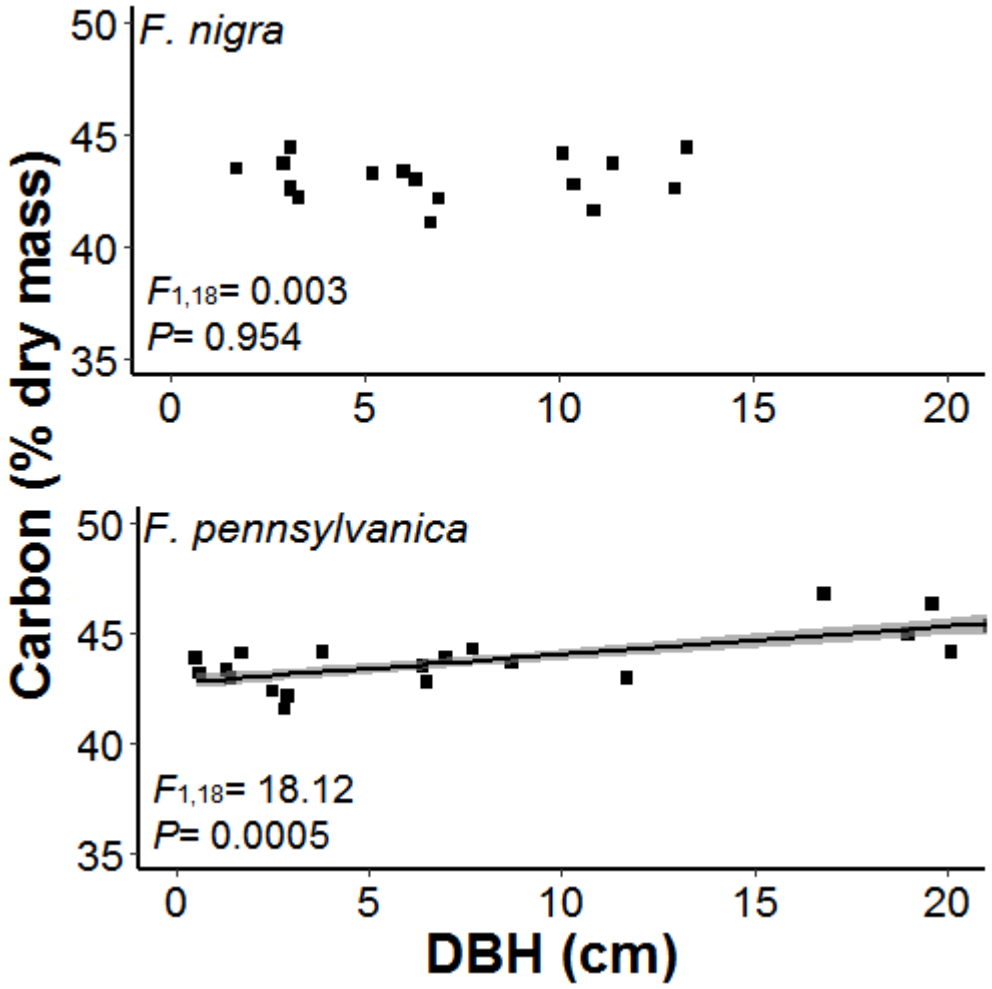

Figure A2-2. Percent carbon of dry mass in leaf tissue vs. diameter at breast height of *Fraxinus nigra* (top) and *F. pennsylvanica* (bottom). Gray ribbon represents standard error of linear regression.

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